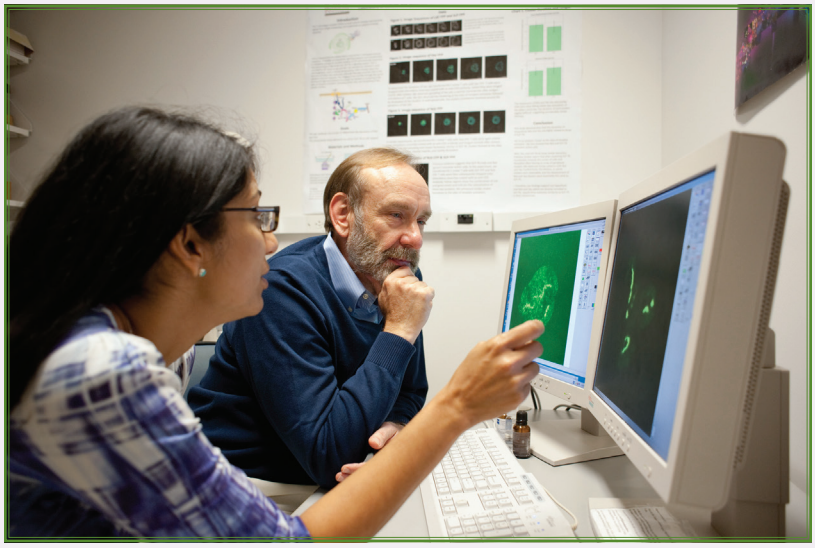
FEATURE

The Complex, Inner Life of T Cells

Among their unique immunological capabilities, T lymphocytes distinguish self from nonself as they patrol the body for invaders. Their acute sensitivity—even a few properly identified molecular intruders can incite them to action—means that their behavior is tightly regulated. The molecular recognition and signaling processes at the heart of the T-cell response have fascinated Lawrence Samelson, M.D., Chief of CCR's Laboratory of Cellular and Molecular Biology, since he chose to do his Yale medical school thesis on immunology in the mid-1970s. Today, his laboratory combines classical biochemistry, genetics, and state-of-the-art imaging to dissect the signaling events that underlie the diversity of T-cell responses at an increasingly fine spatial and temporal resolution. The knowledge they are accruing has contributed fundamental insight into and continues to inform the development of cancer immunotherapies.

(Photo: R. Baer)



Lakshmi Balagopalan, Ph.D., and Lawrence Samelson, M.D.

As a student in Elizabeth Simpson's immunology laboratory in London in 1975, Samelson first learned that T cells simultaneously recognized antigens and the major histocompatibility complex (MHC) marker of "self" through the newly published and ultimately Nobel-prize winning discoveries of Rolf Zinkernagel and Peter Doherty. Seeing the same puzzling result in his own data, Samelson became convinced that he wanted to continue T-cell research after his medical training was completed.

"The T-cell receptor was still a fascinating, mythical creature," explained Samelson.

Samelson joined the laboratory of Ron Schwartz, Ph.D., at the National Institute of Allergy and Infectious Diseases as a Postdoctoral Fellow, trying to generate monoclonal antibodies to a T-cell antigen receptor. "We were one of the early ones to succeed. At that point, I realized I was never going back to medicine. The big question for me was how does the receptor function: how does it recognize an antigen and transmit that recognition inside the cell. And that's what I've been studying ever since."

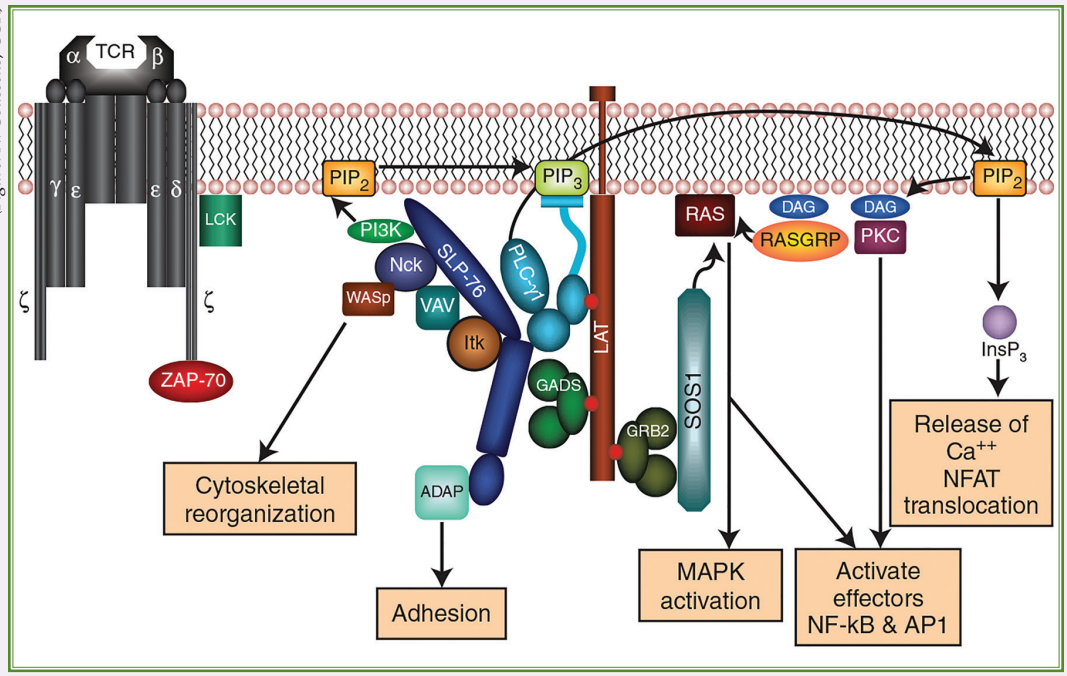
The Basics of T-Cell Receptors

T lymphocytes recognize antigens that are processed and presented to them in combination with MHC molecules by specialized antigen-presenting cells. During their development, individual T cells are selected for survival based on the ability of their receptors to recognize MHC molecules and are selected for destruction if they recognize antigens that are normally present in the body. Mature T cells are specialized to cooperatively mount a complex defense when presented with foreign antigens.

"T lymphocytes do a lot of things. As a cell differentiates in and later outside of the thymus, decisions are imposed by the environment that determine what kind of T cell it is going to be. What kind of cytokines does it make? Where does it go? Many of these complex differentiating events have to do with signaling through the antigen receptor," said Samelson.

T-cell receptors comprise multiple

(Figure: N. Coussens, CCR)



Ligation of the TCR induces tyrosine phosphorylation of numerous adapter and effector proteins leading to the activation of multiple signaling pathways important for gene transcription, cytoskeletal reorganization, and cell adhesion. LAT is central to this process by nucleating multiprotein signaling complexes that are important for enzyme activation and signal propagation.

subunits, which together span the outer membrane to transduce molecular recognition on the cell surface into intracellular action. During a multiyear collaboration with Richard Klausner, M.D., Samelson defined many of these subunits, including the critical ζ subunit.

Like many such transduction events, phosphorylation by tyrosine kinases is a key initiating step. Samelson became interested in the substrates of this phosphorylation and focused on one heavily phosphorylated molecule, which his laboratory finally cloned in 1998 and named Linker for Activation of T cells (LAT).

"LAT is heavily phosphorylated, after which many molecules are recruited to bind it from the cytosol and plasma membrane to create signaling complexes—probably not just one type, but hundreds of them. My underlying assumption is that understanding the complexity of signaling complexes helps you to understand the complexity of T-cell function," said Samelson. His laboratory has done extensive biochemistry to tease apart the binding events and interactions of LAT within a molecular alphabet soup that includes Grb2, Grap, PLC-γ1, and phosphatidylinositol 3-kinase (PI3K). From such studies and analyses of molecular mutations, the field has gradually built a picture linking LAT phosphorylation to pathways for cytoskeletal reorganization, adhesion, calcium influx, and gene expression (see Figure).

Clusters within Clusters

"Another approach that has proven very interesting is to do signal transduction studies through imaging," said Samelson. "Instead of grinding up 10 million cells, you can look at one cell and a few thousand molecules. You can study signaling in time and space."

The Samelson laboratory has employed a variety of imaging capabilities to get the spatial and temporal resolution they need to study T-cell receptor activation. Using confocal imaging and total internal reflection fluorescence (TIRF) microscopy, they have looked at individual T-cell activation events. "Within the first few seconds, as a T cell is activating, a lot of rapid events important for later signaling occur," explained Staff Scientist Lakshmi Balagopalan, Ph.D. "LAT is probably not binding all its associated molecules at once. So speed is important to understand the heterogeneity of responses."

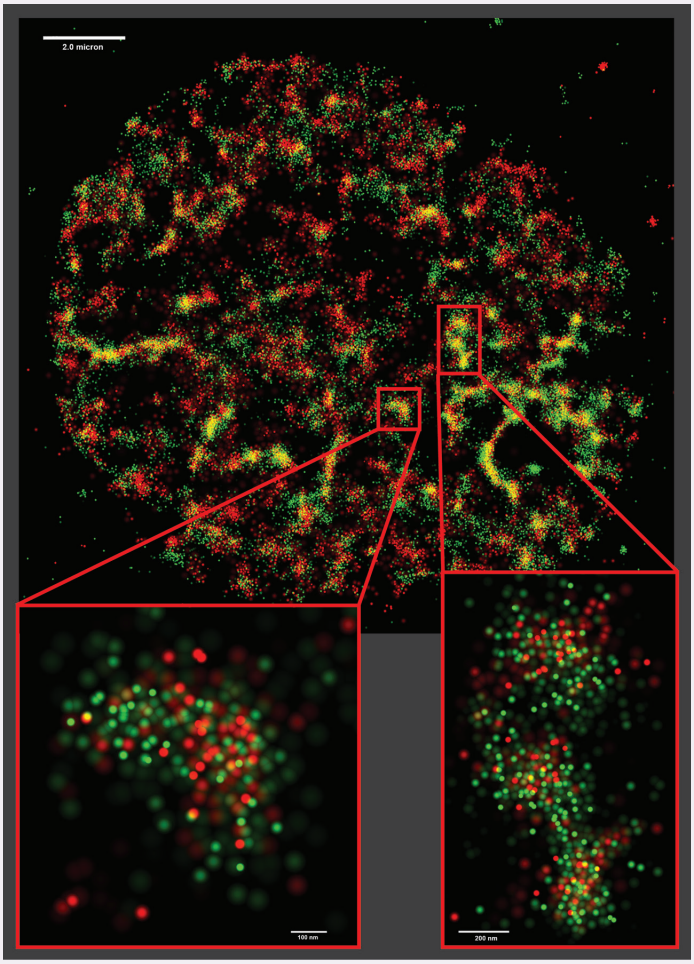
Spatial resolution is also critical. "The big discovery is that these signaling molecules are in microclusters, which are the basic signaling units," said Samelson. Microcluster formation precedes the formation of immunological synapses, which mediate signaling between immune cells.

Initially working with Jennifer Lippincott-Swartz, Ph.D., at the Eunice Kennedy Shriver National Institute of Child Health and Human Development, to establish the technique, Samelson's laboratory

has moved into super-resolution microscopy in order to see into the microclusters. The specific technique, photoactivated localization microscopy (PALM), uses stochastic activation and imaging of rare fluorescent molecules as a means to bypass the normal diffraction limits of light microscopy. PALM has given the team new insights into T-cell receptor organization, and new questions.

"You don't see a homogenous distribution of molecules. Nanoclusters make up the microclusters you can see with confocal microscopy," explained Samelson.

Balagopalan is particularly enthusiastic about a recent collaboration to do focused ion beam scanning electron microscopy (FIB-SEM) with Sriram Subramaniam, Ph.D., Senior Investigator in CCR's Laboratory of Cell Biology. With this technology, they will be able to correlate the confocal images with structural information from electron microscopy. "We are actually going to look at how what we are seeing by fluorescence microscopy correlates with the ultrastructure of clusters."



(Image: J. C. Yi, E. Sherman, and L. Samelson, CCR)

PhotoActivation Localization Microscopy (PALM) was used to image Jurkat T cells activated by contact with a stimulatory surface. This method is able to show the location of proteins with a resolution of 20 nm. Two proteins needed for signal transduction are shown; the green dots show molecules of the adapter LAT, while the red dots show molecules of Grb2. This image shows that these two proteins mix randomly within large microclusters of signaling proteins. Eric Betzig, Ph.D., was awarded the Nobel Prize for Chemistry in 2014 for developing this technique.

Linking to Function

Gathering biochemical, biophysical, and imaging data, Samelson and his colleagues are able to make predictions about the contributions of signaling molecules to T-cell function, which they test *in vivo*. Recently, in a paper in *Science Signaling*, they were able to elegantly separate two distinct functions of the interactions between LAT and its partner, Sos1.

Sos1 is a prototypical guanine nucleotide exchange factor (GEF), which activates the small GTPase Ras by replacing GDP with GTP, thereby initiating a signaling cascade important for cell proliferation. By introducing a point mutation in Sos1, inhibiting its GEF catalytic activity, they showed that Sos1 has an independent ability to act as a scaffold for multiple LAT molecules. This oligomerization—and not the GEF activity—was necessary for optimal T-cell receptor-dependent phosphorylation and activation of calcium signaling in thymocytes. But, both activities were required for normal T-cell development.

"That work is an excellent example of the multiple approaches employed in Larry's laboratory coming together to answer an important question. It started out with a prediction of a former Postdoctoral Fellow, Jon Houtman, Ph.D., now at the University of Iowa, based on isothermal titration calorimetry with little pieces of protein in a test tube, that Sos1 wasn't just acting as a guanine nucleotide exchange factor for Ras in this system," said Balagopalan. "And it ended with a talented postdoc, Rob Kortum, making a series of mutant mice to successfully test this hypothesis."

"Making the transgenic mice took about five years," said Samelson. "Sos1 is one of the granddaddies of the field, but no one had made a conditional knockout. The NCI has this tremendous transgenic mouse facility in Frederick, which made it possible to go through many different transgenes to get sufficiently low levels of expression to minimize artifacts and do all the necessary crosses."

Toward a Better T Cell

The immune system has a special challenge in identifying cancer cells which, after all, are derived from "self". Moreover, cancers evolve rapidly and take active steps to camouflage themselves and subvert immune signaling. Recently, the use of designer T cells, whose receptors have been engineered to recognize cancers, has made some promising advances in the clinic. These chimeric antigen receptors (CAR) are made by coupling an antibody that recognizes a tumor antigen to the T-cell receptor ζ chain, thereby coupling it to the entire T-cell signaling machinery.

"If you focus on the CAR story, you find that bypassing the normal antigen receptor isn't enough. You need to add signaling elements from costimulatory pathways," said Samelson. "Understanding and manipulating the signaling pathways is how immunotherapy will continue to improve."

Progress will no doubt be accompanied by unexpected complexity. Making another point mutation in LAT, Samelson's laboratory found that they were able to make T cells hyperactive and more potent as measured in a number of assays. Early signaling events like calcium influx were greater. A mouse expressing the mutant T cells had increased cytokine production. In another assay, the mutant T cells were able to destroy targets more effectively. "We thought we had made a better T cell," said Samelson.

Working with Nicholas Restifo, M.D., Senior Investigator in CCR's Surgery Branch, Samelson discovered that the story was not so straightforward. "We did a lot of studies to see whether the T cells in our mouse would clear tumors better. And they didn't. The cells were too good; they got overactivated and exhausted."

However, the collaboration with the Restifo lab continues, focused on another knockout mouse model that has improved tumor clearance. Samelson and his colleagues have recently identified the mechanism of action accounting for the superior T-cell response, a prerequisite for developing superior therapies.

To learn more about Dr. Samelson's research, please visit his CCR website at https://ccr.cancer.gov/lawrence-e-samelson.

Scientists without Borders

The Laboratory of Cellular and Molecular Biology (LCMB), of which Lawrence Samelson is Chief, comprises six research groups working on diverse questions in signal transduction, ranging from receptor activation to nuclear transport. When the laboratory moved to its current facilities, Samelson ordered that the doors between the labs be taken down.

"Some people were skeptical, but it has really been transformative in the free flow of traffic between labs. He has literally taken down the barriers between us!" said Staff Scientist Lakshmi Balagopalan, Ph.D. "The Samelson group and LCMB are by far the most open and congenial environments I have ever been in; and, a large part of the credit for this goes to Larry."

Balagopalan joined Samelson's laboratory as a Postdoctoral Fellow in 2003. Trained as a fly geneticist, she was looking to switch into immunology. She did some homework, interviewing current and former members of the lab, who gave strong positive recommendations. "Larry is a fantastic mentor, who creates a great environment conducive to creativity and research. No politics, just focus on the work." Senior lab members like Balagopalan and fellow Staff Scientist Connie Sommers, Ph.D., are encouraged to take senior authorship on manuscripts. "That is a big motivator for us to drive and take ownership of projects."

The strength of the immunology field in CCR and the NIH Intramural Program is tremendous including multiple institutes, like the National Institute of Allergy and Infectious Diseases, and the National Heart, Lung, and Blood Institute. "It's a tight community," said Balagopalan. "When I entered the field, it was like learning a new language. I took an immunology course that Larry encourages incoming trainees to take run by the Foundation for Advanced Education in the Sciences. It's an amazing introduction, taught by leaders in the field like Ron Germain, Bill Paul, and of course, Larry."

"The kind of materials we use cannot be bought on the street. Experts here can make phosphorylated 80-amino acid peptides. Experts here are world leaders in analytic techniques and high-resolution microscopy. And I can't think of a place in the world where there are more outstanding immunologists," said Samelson. "The NIH Intramural Research Program makes it possible to do the work we do."